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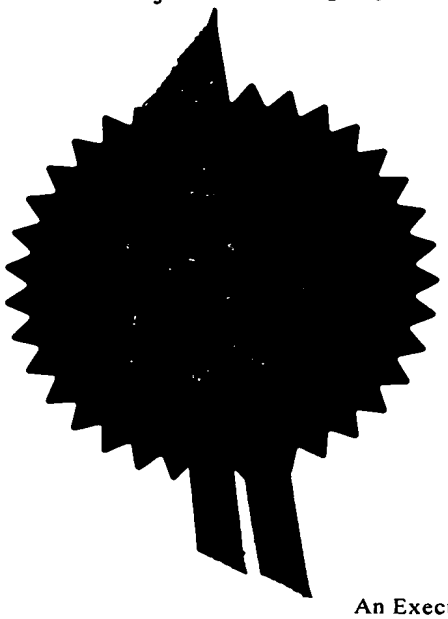

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08JUL98 E374016-1 D00354
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| 2. | Patent application number *(The Patent Office will fill in this part)* | **9814715.0** |
| 3. | Full name, address and postcode of the or of each applicant *(underline all surnames)* | ~~VUMAN LIMITED~~ The Victoria University of Manchester<br>RUTHERFORD HOUSE<br>MANCHESTER SCIENCE PARK<br>PENCROFT WAY<br>MANCHESTER<br>M15 6SZ<br>OXFORD ROAD<br>MANCHESTER<br>M13 9PL |
| | Patents ADP number *(if you know it)* | 773945001 |
| | If the applicant is a corporate body, give the country/state of its incorporation | |
| 4. | Title of the invention | FLUORESCENCE ANALYSIS |
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# Fluorescence Analysis

This invention relates to a method and apparatus for fluorescence analysis.

Fluorescence analysis is an important experimental tool for both chemists and biologists, and is of particular interest to pharmaceutical researchers.

A known method of analysing fluorescence comprises exciting a sample of many hundreds of fluorophores with an intense pulse of light from a laser. The intensity of fluorescence emitted by the sample at a given delay following excitation is detected. The sample is excited again, and the intensity of fluorescence emitted by the sample at a different delay following excitation is detected. A series of measurements at various delays are made, and the fluorescence intensity is plotted as a function of the delay, to provide a distribution of fluorescence intensity over time. An upper state lifetime characteristic of the fluorophores comprising the sample may be derived from the gradient of the intensity distribution (for example, by fitting an exponential decay curve to the distribution).

A limitation of the above method is that it requires a sample of many hundreds of fluorophores and high intensity illumination. A sample of the order of 100 fluorophores will not provide sufficient signal to noise to allow the measurement of fluorescence. The method cannot therefore be used to measure effects caused by a single fluorophore or a sample containing a small number of fluorophores or a sample that optically quenches rapidly. Furthermore, the method requires the use of pulsed laser sources and gated detectors to measure time domain fluorescence parameters such as decay rate.

It is an object of the present invention to provide a method and apparatus for the detection of fluorescence which is capable of measuring fluorescence from a sample of several fluorophores.

According to a first aspect of the invention there is provided a method of fluorescence analysis for determining a characteristic lifetime of a sample, the method comprising exciting the sample either continuously or using pulses having an inter-pulse separation which is of the order of or less than the characteristic lifetime of the

sample, detecting photons emitted by the sample to obtain a detected signal, correlating the detected signal with itself and analysing the correlated signal to derive the characteristic lifetime, wherein the number of fluorophores in the sample and the intensity of the excitation are such that photons are detected in a stream in which individual photons are distinguishable from each other.

Correlation in the above context includes performing a Fourier transform, using a Fabry-Perot etalon, or any other suitable signal processing.

Preferably, the excitation is of sufficient intensity that each fluorophore is excited substantially immediately after photon emission.

Where the excitation is not of sufficient intensity that each fluorophore is excited substantially immediately after photon emission, analysis of the detected signal preferably includes the determination of the rate of photon collisions with each fluorophore, thereby allowing the characteristic lifetime to be determined.

Preferably, the sample is excited by illumination, most preferably using a laser beam.

The sample preferably comprises less than 100 fluorophores, and may comprise less than 10 fluorophores.

Preferably, the method further comprises selecting the number of fluorophores and intensity of excitation so as to maximise the signal-to-noise ratio of the detected signal.

According to a second aspect of the invention there is provided a fluorescence analysis apparatus for determining the characteristic lifetime of a sample, comprising means for exciting the sample either continuously or using pulses having an inter-pulse separation which is of the order of or less than the characteristic lifetime of the sample, means for detecting photons emitted by the sample to obtain a detected signal, correlating means for correlating the detected signal with itself and analysing means for analysing the correlated signal to derive the characteristic lifetime, wherein the number of fluorophores in the sample and the intensity of the excitation are such that photons are detected in a stream in which individual photons are distinguishable from each other.

A specific embodiment of the invention will now be described by way of example, with reference to the accompanying drawings, in which:

Figure 1 is a schematic representation of a fluorescence detection apparatus according to the invention;

Figure 2 is a set of graphs which illustrate the operation of the invention for a very small number of fluorophores; and

Figure 3 is a set of graphs which illustrate the operation of the invention for a larger number of fluorophores.

Referring first to Figure 1, a fluorescence detection apparatus comprises a sample 1 and a laser 2 which is arranged to illuminate the sample 1 with a continuous beam of light. A detector 3 is provided to detect photons emitted by the sample 1. The detector 3 may be provided with a filter (not shown) to prevent the detection of light emitted by the laser 2. An auto-correlator 4 correlates the detected signal to give a correlation, and a processor 5 processes the output of the detector to measure the characteristic lifetime of the fluorophores comprising the sample 1. The operation of the processor 5 is described below. The output from the processor 5 and/or the auto-correlator 4 may be displayed on a monitor 6.

Figure 2 illustrates the principle of operation of the invention. Figure 2a represents the result of illuminating a single fluorophore with a continuous beam of high intensity light. A photon in the high intensity beam will excite the fluorophore to an excited state, and following a time period characteristic of the fluorophore, the fluorophore will emit a photon (i.e. it will fluoresce) and return to its unexcited state. Since the fluorophore is being illuminated by a continuous stream of photons from the laser, once it has returned to its unexcited state it will be excited again immediately to its excited state. The fluorophore will again emit a photon following a characteristic time period. Thus, a series of photons will be emitted by the fluorophore, each photon being separated from the previous photon by a time period characteristic of the fluorophore, as a illustrated in Figure 2a.

In Figure 2a, signals corresponding to each photon emission are shown as being equally spaced. In fact, the interval between fluorophore excitation and photon

emission will not be constant but will vary about a mean in a manner characteristic of the fluorophore being excited. Such variations are taken account of however by the auto-correlation process described above. Thus for the purposes of illustration only Figures 2a to 2c have been prepared on the assumption that the excitation to photon emission interval is constant.

The graph shown in Figure 2a illustrates an ideal scenario in which the sample comprises a single fluorophore, that every photon emitted by the fluorophore is detected, that the fluorophore is excited immediately when it decays to its unexcited state, that the fluorophore cannot be further excited when it is in the excited state, and that no light from the laser is detected.

Figure 2b illustrates a second scenario, where the sample comprises two fluorophores of a single type, and all of the other assumptions given above still apply. In this case, each fluorophore will emit a series of photons as above. The characteristic lifetime of the fluorophore is apparent from the separation of the photons in each series, as an auto-correlation of the photon distribution will provide a strong peak at a frequency which corresponds to the characteristic lifetime of the fluorophore.

Figure 2c illustrates a third case which corresponds to that shown in Figure 2b, except that not all of the photons emitted by the fluorophore are detected. Although not all of the emitted photons are detected, the number detected is sufficient that, an auto-correlation of the signal of Figure 2c will yield a peak at the frequency which corresponds to the characteristic lifetime of the fluorophore, and there will now be a further peak at two times that frequency, a peak at three times that frequency, and so on.

Thus, it is not necessary that every photon emitted by a sample of fluorophores is detected, but rather all that is required is that the number of photons detected is sufficient for an auto-correlation of the detected signal to yield measurable peaks at multiples of the frequency which characterises the sample of fluorophores.

There is an upper limit to the number of fluorophores which may be included in a sample. If for example a sample comprised more than 100 fluorophores, and the

detector detected all photons emitted by the fluorophores, then the number of photons would be so great that the detected signal would become 'washed out' and it would not be possible to obtain any information from the signal.

As stated above, in an ideal scenario a single fluorophore only would be measured. However, in practice the assumptions made for that ideal scenario will not hold. For example, not all of the photons emitted by the fluorophore will be detected. Furthermore, not all of the fluorophores included in a sample will be active. To maximise the signal-to-noise ratio of the detected signal, an optimum combination of sample size and intensity of laser illumination may be selected. The selection will depend upon the specific optical arrangement used, and will be influenced by, for example, the efficiency of detection of photons and the quantum efficiency of the fluorophore sample. Thus, the number of fluorophores to be used is determined experimentally by maximising the signal-to-noise ratio of the detected signal for given experimental conditions. The ideal signal level will correspond essentially to the observation of a single fluorophore in the ideal scenario as described above, although this will actually involve detecting a percentage of the emission of between approximately 5-50 fluorophores.

Figure 3 illustrates the operation of the invention close to its upper limit (in terms of intensity of detected light). If apparatus as shown in Figure 1 is used to detect photons emitted by a sample of a large number of fluorophores, for example 50, a detected signal as shown in Figure 3a may be obtained. Rather than detecting individual photons separated by time delays, the number of fluorophores is such that the intensity of detected fluorescent light never falls to zero. Although the detected signal appears to be random, from the above it will be understood that an auto-correlation of the signal will yield a series of peaks which are multiples of a frequency which corresponds to the characteristic lifetime of the fluorophore, thus allowing the fluorophore to be characterised. This is further illustrated with reference to Figure 3b, where again the detected signal appears to be random. Assuming that the sample from which the symbols of Figure 3a and 3b are derived include the same number of fluorophores, the signal shown in Figure 3b corresponds to a fluorophore with a

characteristic lifetime which is ten times that of the lifetime shown in the signal of Figure 3a. Although both Figures 3a and 3b appear to have random signals, it is apparent from the number of times that the signal crosses its mean value that the characteristic lifetime of the sample detected in Figure 3b is less than that of the sample detected in Figure 3a.

The above described embodiment assumes that the intensity of light incident on a sample is sufficient such that once a given fluorophore has returned to its unexcited state it will immediately be excited to its excited state. This is the optimum experimental set-up since a detected signal will be a function only of the characteristic lifetime of the fluorophore. If a lesser intensity of incident light was used, then the separation of the photons emitted from a given fluorophore would be a function of the characteristic lifetime of the fluorophore and the frequency of photons colliding with the fluorophore. The frequency of photon collision will be constant for a given laser intensity and experimental set-up, and may be removed from a detected signal.

The laser shown in figure 1 is described as being arranged to illuminate a sample with a continuous beam of light. This is an advantageous arrangement, since continuous wave (CW) lasers are cheap and widely available. However, it will be understood that the sample may be illuminated using pulsed light. When pulsed light is used the interpulse separation must be of the order of or less than the characteristic lifetime of the sample fluorophores. If the interpulse separation were to be greater than this then the amount of time spent by each fluorophore in an unexcited state would introduce noise into the detected signal to such an extent that fluorophore lifetime measurement would be compromised.

It will be understood that the characteristic lifetime of a fluorophore may be derived from the detected signal in several ways, including using signal analysers, analogue correlators, storage and software correlation or Fourier transforms.

The invention may be used to study the quantum efficiency of fluorophores. Quantum efficiency is a measure of the number of photons emitted by a fluorophore in relation to the number of photons 'absorbed' by that fluorophore.

Generally, a photon will, upon absorption by a fluorophore, excite that fluorophore to a state from which it will relax to an unexcited state by the emission of a photon (this is fluorescence). However, some excited states of a fluorophore will not allow the emission of a photon, and the excitation must be dissipated in some other way, for example as heat. A 'non-fluorescent' excited state may or may not be capable of being excited further to a 'fluorescent' excited state by absorption of a further photon. Known prior art fluorescence detection apparatus requires a sample of many fluorophores, and it is not possible to perform a full study of the quantum efficiency of a given type of fluorophore, since the effect of excitation to 'non-fluorescent' states is averaged out for all fluorophores in the sample. The invention allows the study of a sample comprising a very small number of fluorophores, which thereby allows more detailed investigation of 'non-fluorescent' states.

## Claims

1. A method of fluorescence analysis for determining a characteristic lifetime of a sample, the method comprising exciting the sample either continuously or using pulses having an inter-pulse separation which is of the order of or less than the characteristic lifetime of the sample, detecting photons emitted by the sample to obtain a detected signal, correlating the detected signal with itself and analysing the correlated signal to derive the characteristic lifetime, wherein the number of fluorophores in the sample and the intensity of the excitation are such that photons are detected in a stream in which individual photons are distinguishable from each other.

2. A method according to claim 1, wherein the excitation is of sufficient intensity that each fluorophore is excited substantially immediately after photon emission.

3. A method according to claim 1, wherein the excitation is not of sufficient intensity that each fluorophore is excited substantially immediately after photon emission, and analysis of the detected signal includes the determination of the rate of photon collisions with each fluorophore, thereby allowing the characteristic lifetime to be determined.

4. A method according to claim 1, 2 or 3, wherein the sample is excited by illumination.

5. A method according to claim 4, wherein the sample is illuminated by a laser beam.

6. A method according to any preceding claim, wherein the sample comprises less than 100 fluorophores

7. A method according to claim 6, wherein the sample comprises less than 10 fluorophores.

8. A method according to any preceding claim, further comprising selecting the number of fluorophores and intensity of excitation so as to maximise the signal-to-noise ratio of the detected signal.

9 A fluorescence analysis apparatus for determining the characteristic lifetime of a sample, comprising means for exciting the sample either continuously or using pulses having an inter-pulse separation which is of the order of or less than the characteristic lifetime of the sample, means for detecting photons emitted by the sample to obtain a detected signal, correlating means for correlating the detected signal with itself and analysing means for analysing the correlated signal to derive the characteristic lifetime, wherein the number of fluorophores in the sample and the intensity of the excitation are such that photons are detected in a stream in which individual photons are distinguishable from each other.

10. A method of fluorescence detection substantially as hereinbefore described with reference to the accompanying figures.

11. A fluorescence detection apparatus substantially as hereinbefore described with reference to the accompanying figures.

## Abstract

A method of fluorescence analysis for determining a characteristic lifetime of a sample. The method comprises exciting the sample either continuously or using pulses having an inter-pulse separation which is substantially less than the characteristic lifetime of the sample, and detecting photons emitted by the sample to obtain a detected signal. The detected signal is correlated with itself and analysed to derive the characteristic lifetime of the sample. The number of fluorophores in the sample and the intensity of the excitation are such that photons are detected in a stream in which individual photons are distinguishable from each other.

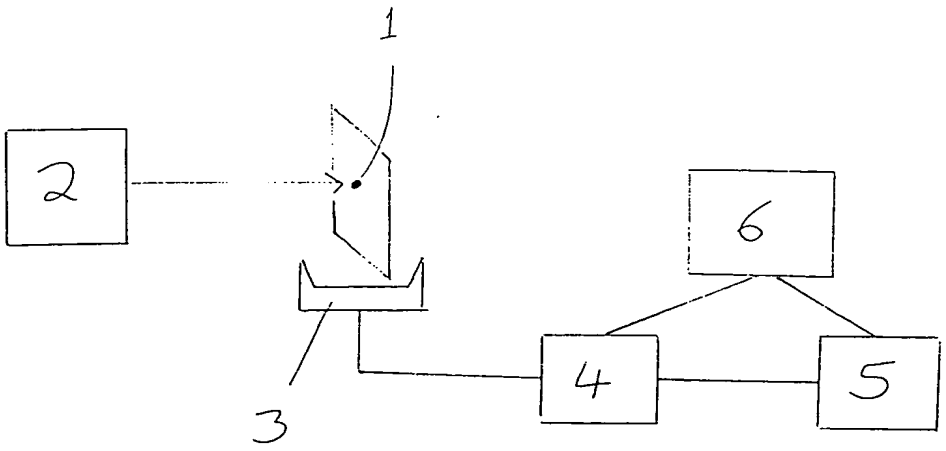


FIG. 1

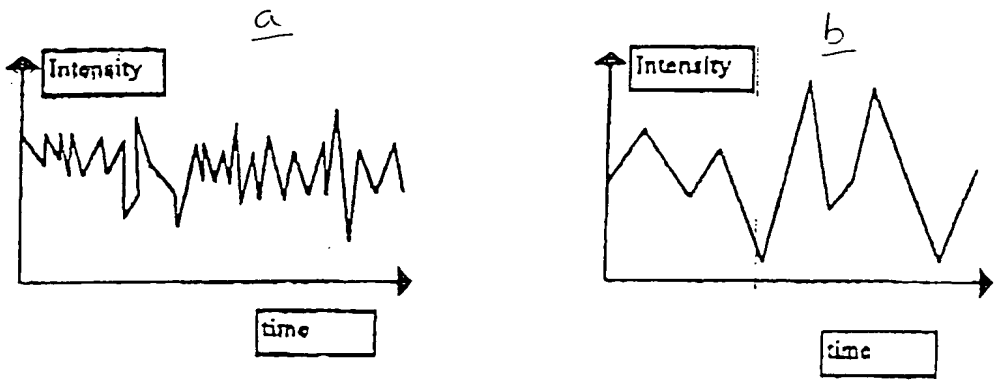


FIG. 3

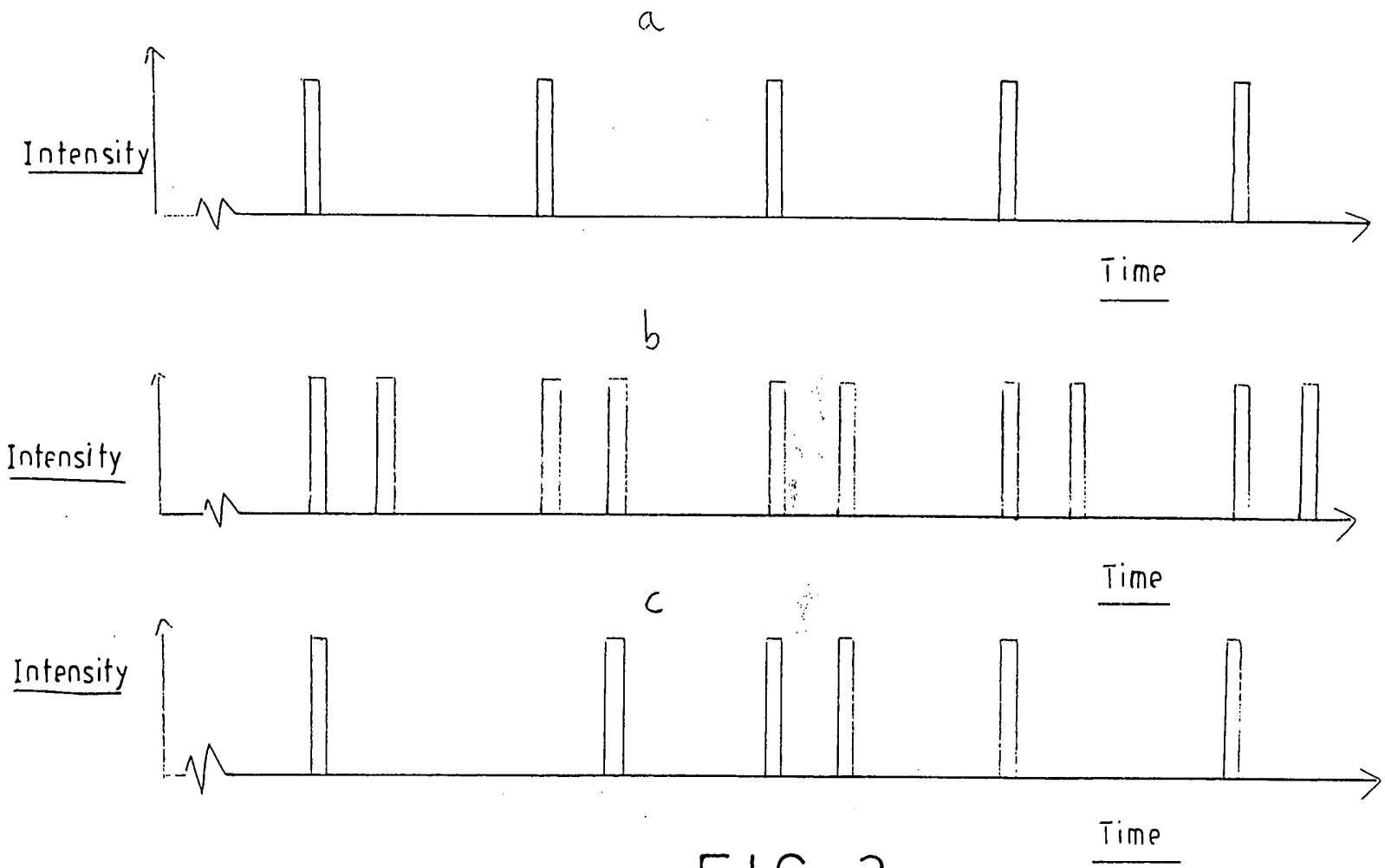


FIG. 2

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